QUANTITATIVE AGREEMENT BETWEEN THE VALUES FOR THE LIGHT-INDUCED ΔpH IN *RHODOPSEUDOMONAS SPHAEROIDES* MEASURED WITH AUTOMATED FLOW-DIALYSIS AND ^{31}P NMR

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1. Introduction

Quantitative determination of transmembrane pH and electrical potential gradients is a prerequisite for a further refinement of the concepts of the chemiosmotic hypothesis [1]. Several methods are available for the measurements of ΔpH and $\Delta\psi$ (review [2]), of which the 'spectroscopic' and 'distribution' methods are most widely used. The outcome of the various methods, however, does show significant variations and it has been established by stringent comparisons, that the optical methods overestimate the magnitude of the transmembrane gradients [3–6]. The distribution methods [2,7] (except flow-dialysis [8]) have the disadvantage that leakage of the probe molecules may occur during the separation step. Furthermore, in any distribution method uncertainties remain concerning:

(i) The homogeneity of the internal aqueous phase and the absence of subcellular compartments;
(ii) The 'ideal behaviour' [2] of the indicator probe;
(iii) The activity coefficient of the probe molecules in the internal aqueous phase.

With the application of ^{31}P NMR to biological systems [9–11] a powerful and independent method has become available for the quantitation of ΔpH. This technique makes use of the pH dependence of ^{31}P NMR chemical shifts of phosphate metabolites. It can be used only if calibration curves of the chemical shift of intracellular and extracellular phosphate metabolite versus pH are available. For the evaluation of the magnitude of ΔpH with the use of ^{31}P NMR, two assumptions have to be made:

(i) Zero ΔpH in the absence of energy supply and in the presence of an uncoupler;
(ii) A phosphate metabolite is present in a significant concentration in the compartment of interest.

Obviously, strong support for the validity of ΔpH determinations is supplied when the same ΔpH values are obtained with flow-dialysis and ^{31}P NMR. A good correlation between the two methods has been reported [12,13]. However, as that comparison was performed under comparable but not identical conditions (see section 4) we decided to reinvestigate this point, especially as it is known that both the ionic composition of the medium [14] and the energy source [7,15] can have a pronounced effect on the magnitude of ΔpH.

Here, we demonstrate that the ΔpH values determined under identical conditions in *Rhodopseudomonas sphaeroides* with ^{31}P NMR and automated flow-dialysis are identical. This conclusion is based on a series of measurements performed at various external pH values and in two media with different ionic composition.

2. Experimental

2.1. *Growth and harvesting of cells*

Rhodopseudomonas sphaeroides was grown [16] and harvested [11] as described, except that the following media were used to wash the cells:

(A) Glycyl–glycine (20 mM), 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid, 20 mM

Abbreviations: ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical potential gradient; SF-6487, 3,5-di-*tert*-butyl-4-hydroxybenzylidine-malono-nitrite

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2-(*N*-morpholino)-ethanesulphonic acid, 2 mM EDTA, 5mM $MgSO_4$, 10 mg chloramphenicol/l, 67 mM Na_2SO_4, brought to the desired pH value with NaOH.

(B) The same as medium (A) except that 100 mM KCl replaced the Na_2SO_4 and KOH was used to adjust the pH of the buffer.

2.2. *ΔpH measurements*

ΔpH measurements were performed with ^{31}P NMR [11] and automated flow-dialysis [3,17] as described. With both techniques, the condition of light-saturation was ascertained through a series of dilution experiments (see section 3) and the presence of 5 μm SF-6847, without illumination, served as the reference condition, in which pH is assumed to be zero. The internal water volume of the cells was measured to be 2.3 μl/mg protein with the use of ^3H_2O and [*carboxyl*-^{14}C]-dextran and according to published procedures (e.g., [18]).

2.3. *Protein determination*

Protein was determined according to [16], after boiling of the intact cells for 2 min in 2 M NaOH.

2.4. *Materials*

[^{14}C]Benzoic acid (108 Ci/mol) and ^3H_2O (91 mCi/mol) were obtained from the Radiochemical Centre (Amersham, Bucks). [*carboxyl*-^{14}C]Dextran (2.8 mCi/g) was obtained from New England Nuclear Corporation (Boston, MA). SF-6847 was a gift from Dr Y. Nishizawa (Sumitomo Chemical Industry, Osaka). All other chemicals were reagent grade and obtained from commercial sources.

3. Results

In order to measure an internal pH from the pH-sensitive resonance position of an internal phosphate metabolite, a titration curve of the compound in the relevant pH range is required. As two different media were used to compare the measurement of the light-generated ΔpH in *Rps. sphaeroides*, this titration was performed in a suspension of cells, incubated in each of these two media. Fig.1 shows the result of the titration in medium A (for the composition of this medium: see section 2). Values of 6.70 and 6.50 for the pK_2 of external and internal P_i were obtained, respectively. The titrations were performed in the presence of 5 μM

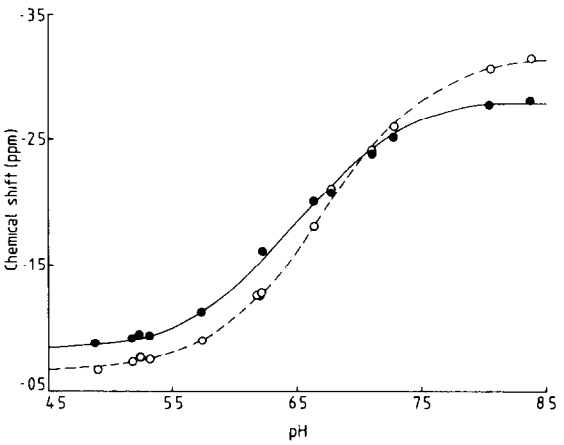

Fig.1. pH-titration curves for intracellular (•) and extracellular (○) P_i in a cell suspension in medium A. The titration was performed as in [11]. The curves drawn were calculated for: (i) intracellular P_i: pK_2 = 6.50 and limiting chemical shifts at low and high pH of −0.83 and −2.84 ppm, respectively; (ii) extracellular P_i, with corresponding values of 6.70, −0.66 and −3.17.

of the uncoupler SF-6847 and in the dark, in order to eliminate a pH gradient across the membrane during this titration [20]. The total change in chemical shift, upon protonation of external phosphate is much larger than the corresponding change of the resonance position of internal inorganic phosphate. This gives rise to intersecting titration curves.

The values for the pK_2 of external and internal P_i in a cell suspension in medium B are 6.69 and 6.73, respectively [11]. In this case the two titration curves do not intersect, but the curve of the external phosphate is shifted slightly to somewhat more negative shift positions, especially in the high pH region.

In the flow-dialysis experiments, light saturation was ascertained through a series of experiments at various light intensities and cell densities [21]. The same procedure in the NMR experiments had to be restricted to a more limited region of cell-densities because the accuracy of the determination of the resonance position of internal inorganic phosphate decreased drastically in cell suspensions with a density <10 mg protein/ml. Therefore the condition of light saturation in these experiments was inferred from an extrapolation of the ΔpH to zero cell density (see fig.2). The extrapolation was executed in two different ways:

(i) In a plot of ΔpH against cell density [21];

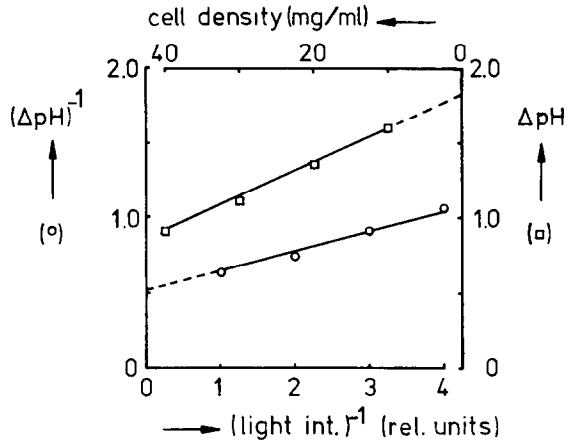

Fig.2. Determination of the ΔpH in *Rps. sphaeroides* at light saturation. The ΔpH in *Rps. sphaeroides* was measured with the use of ^{31}P NMR at a range of cell densities (10–40 mg protein/ml) in medium B at pH 5.4. The results were plotted as described in the text. The extrapolated values for the ΔpH are 1.93 in the plot of 1/ΔpH against 1/light intensity and 1.83 in the plot of cell density against ΔpH.

(ii) In a plot of 1/ΔpH against 1/light-intensity [21–23].

The latter method is based on the light-dependent saturation of the ΔpH and assumes an inverse relation between cell density and light-intensity in the well-stirred sample [11]. In all cases one of the measured values was within 80% of the extrapolated value and usually 4 dilutions were used at a specific external pH value. In the example given in fig.2, the two methods give a value of 1.83 and 1.93, respectively, for the light-dependent ΔpH in *Rps. sphaeroides* in medium B and at an external pH of 5.4.

Fig.3 shows a summary of all the data available on the comparison of ΔpH, as measured with the two independent methods. Clearly, the magnitude of the ΔpH is much larger in the potassium-containing medium, compared to the sodium-containing medium. Most probably this is due to the presence of a sodium/proton-exchange system, which is present in the cytoplasmic membrane of this bacterium [24]. However, in both media an excellent agreement between the two methods is observed (compare open and closed symbols): within 0.1–0.2 pH units the ΔpH values determined with both methods are the same. The absence of a light-induced pH gradient in medium A from pH 6–8, as indicated by the benzoic acid distribution was confirmed with the use of methylamine. A light-

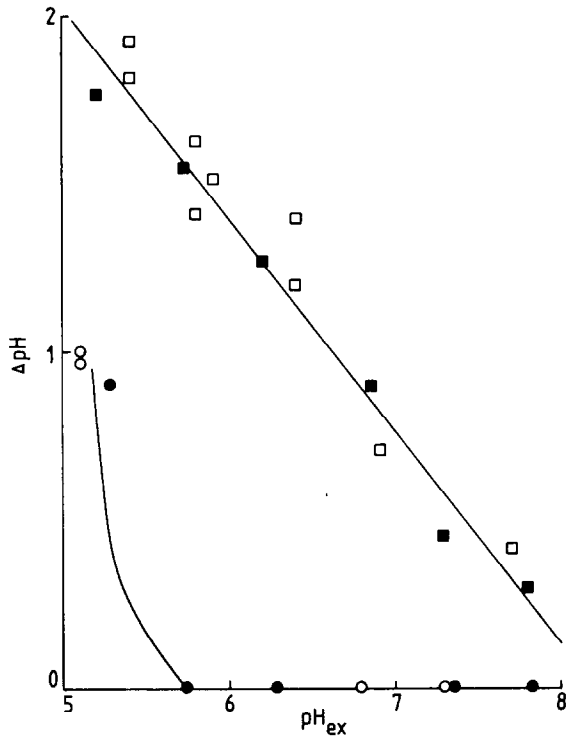

Fig.3. Comparison of the ΔpH in *Rps. sphaeroides*, as measured with automated flow-dialysis and ^{31}P NMR. The two media, described in section 2, were used. In the flow-dialysis experiments, light saturation was ascertained through a series of experiments at various light intensities and cell densities [21]. The concentration of [^{14}C]benzoic acid was 30 μM: (open symbols) ^{31}P NMR; (closed symbols) automated flow-dialysis; (squares) medium B; (dots) medium A.

dependent accumulation of this weak base was also not observed under these conditions. From fig.3 the dependence of the intracellular pH on the external pH in the two media can be calculated. It turns out (fig.4) that the internal pH of *Rps. sphaeroides* does not remain constant in either of the two media, the largest variations occurring in the sodium-containing medium.

It should be noted that the time dependence of the generation of the ΔpH differed considerably, between the measurements at the various external pH values. Indicated are, however, the steady state values. For example, with flow-dialysis in the first 2 or 3 min after the start of the illumination a small but significant ΔpH was generated in medium A, around pH 6, which disappeared in ~10 min. Furthermore, in medium B sometimes a small damped oscillation was

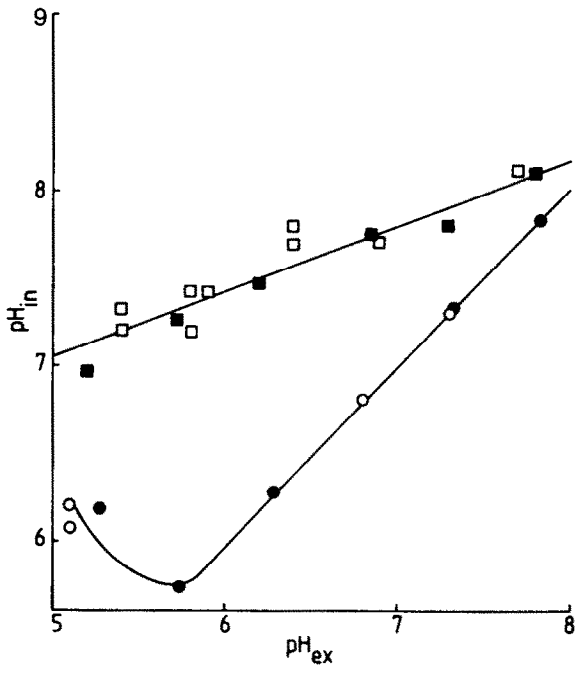

Fig.4. The internal pH in energized *Rps. sphaeroides* cells as a function of the external pH. The data were calculated from fig.3; the same symbols were used.

observed. This point will be elaborated elsewhere (K. J. H., J. L., W. N. K., in preparation). An alternative explanation for the inequality of the chemical shift positions of internal and external P_i after equilibration in the dark and in the presence of 5 μM SF-6847 is the existence of a Donnan potential. Although we cannot rule out this possibility, we consider it unlikely in view of the high ionic strength of the media that we used and the large permeability of, e.g., Cl^- in one of these media. Although such a Donnan potential would cause a change in the absolute pH-values that have been determined, it does not weaken our conclusion concerning the comparison of the two methods, as the same reference situation was used in flow-dialysis and NMR experiments.

4. Discussion

The pK_2 values of P_i in the two media are very similar, as one would expect from their close correspondence in composition and ionic strength. By contrast, inside the cells the two pK_2 values differ significantly. Possibly, the shift of the pK_2 value in medium A can be explained by an increase in ionic strength inside the cells in this medium [25]. The ionic strength does not have an effect on the chemical shift at the extreme pH values, but the intersection of the titration curves in fig.1 can be caused by residual paramagnetic cations, which interact more strongly with P_i at the higher pH values [11].

Although light of saturating intensity was used in the two experimental set ups, the wavelength distribution differed significantly [11,17]. From action spectra of light-dependent proton extrusion from intact cells of *Rps. sphaeroides*, in the presence of valinomycin, it is clear that the maximal response upon light saturation is independent of the wavelength of the actinic light, between 400 and 900 nm (W. de Vrij, K. J. H., unpublished).

Comparison of ^{31}P NMR and 'distribution' measurements to quantitate energy-dependent pH gradients has also been reported in other systems. In [34, 35] these two methods were compared in chromaffin granules. The two methods were concluded to give similar results for the internal pH of the granules. A quantitative comparison of the results in this system is complicated by the calibration of the shift position of the internal probe in the NMR method [34–36]. In a suspension of rat liver mitochondria, energized by the oxidation of endogenous substrates and with a ΔpH of 0.5 pH units, the two methods agree within 0.1 pH unit [25]. Finally, in [12,13] in *Escherichia coli* ^{31}P NMR and silicon oil centrifugation [26] gave the same value for ΔpH. Close inspection of the data shows that:

(i) At pH 7 values of −54 mV ([13]; fig.4) and −37 mV (table 1 in [26]) were obtained, respectively. Furthermore strain MRE 600 and succinate as carbon source were used in [13], in contrast to strain K12 and 43 mM glycerol in [26];

(ii) At pH 6 the values for ΔpH are −83 to −103 mV in the ^{31}P NMR experiment ([12]; fig.3) and a value of −118 mV is obtained with silicon oil centrifugation (table 5 in [26]). Here again the two different strains were used, whereas glucose (3 mM) and succinate (16 mM) served as energy source, respectively.

A similar comparison of the quantitation of $\Delta\psi$ by two independent methods has also been reported. Comparison of measurements with flow-dialysis and with microelectrodes has resulted in the conclusion that the two methods show an excellent agreement [37,38]. Such a comparison cannot be made, however,

in smaller systems (like bacteria) which are not amenable to microelectrode injection. Therefore, it would be desirable to have an alternative method available to measure the $\Delta\psi$ in those systems. Possibly, an analogous NMR method for these measurements can be designed.

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